## Example 14

### Polymyxin-B Sulfate Removal of Endotoxin

1. Material Preparation (all buffers/materials prepared with pyrogen-free $H_2O$ and pyrogen-free glassware):

PHMA media in 10 ml columns

a. wash with 20 ml $H_2O$
b. 1.5% $NaIO_4$, in $H_2O$, passed through at 1 ml/min for 30 min
c. wash with 20 ml $H_2O$
d. equilibrate with 0.1M Sodium Phosphate buffer, pH 7.5
e. 6 mls of a 5 mg/ml Polymyxin-B Sulfate solution in above buffer is recirculated overnight (0.5 mls/min)
f. uncoupled Polymyxin-B Sulfate washed out with 0.1M Phosphate buffer (saved for determination of amount coupled)
g. column then equilibrated with 0.1M Borate buffer, pH 8.0
h. 20 mls of a 1% ethanolamine solution recirculated with 4 x 25 mg $NaBH_4$ additions added 60 minutes apart
i. column washed with 0.1M NaP, pH 7.5 buffer and 0.15M HCl + 0.25M NaCl, pH 2.7, solution alternately (20 mls each; 2 times for each solution)
j. column equilibrated with 0.1M NaP buffer pH 7.5 [for a PHMA 250 ml (12.5 g of media) cartridge--same procedure with an 75X scale-up]

The 10 ml column is the mini-column with a 10 mm diameter.

2. Testing Procedure

a. 0.02M NaP + 0.15M NaCl pH 7.0 + 10 mg/ml E. coli LPS (Sigma No. L-2880 lipopolysaccharide from E. coli 05S:B5 phenol extract) flowed at 1 ml/min for 10 ml cartridge and 20 ml/min for 250 ml cartridge.
b. Same as a. except 20 mg/ml BSA added to buffer.

3. Assay Methods for Pyrogen Levels

Test used: Whittaker Bioproducts' QCL1000 Quantitative Chromogenic LAL assay.

Principles of this test: Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the Limulus Amebocyte Lysate (LAL). The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyzes the splitting of p-nitroaniline (pNA) from a colorless substrate and the yellow color is measured photometrically at 405 nm after the reaction is stopped with acetic acid. After standards are run, the correlation between the absorbance and the endotoxin concentration is linear in the 10-200 pg/ml range.

Application of this test: The starting material is diluted with sterile saline to the proper concentration range and assayed to form a curve. The unknown samples, diluted approximately the same way, are assayed against this curve. The pg/ml value obtained for the unknown, times the dilution equals the concentration of endotoxin in the unknown.

4. Results

a. Coupling efficiency: When 60 mg of Polymyxin-B Sulfate/gram of activated PHMA is given, 26-31 mg/gram can be coupled.

b. LPS binding capacity (everything dependent on LPS concentration)

In test procedure A:

10 ml cartridge
(700-1000) mg/g media
33.5 µg/mg Polymyxin-B Sulfate

250 ml cartridge 43.7 µg/ml PMBS
530 µg/g media

In test procedure B:

| | |
|---|---|
| 10 ml cartridge | 26.5 µg/mg PMBS |
| 250 ml cartridge | 32.1µg/mg PMBS |

## Example 15

### Protein A and Protein G Affinity Filters Configured for Radial Flow

Immobilized recombinant Protein A and Protein G were used as model proteins to demonstrate the functions of the affinity separation media. Protein A is a well-known staphylococcal-derived protein that interacts with Fc region of immunoglobulins, especially immunoglobulin G. Uses of Protein A include the detection and purification of antibodies and immune complexes, bulk production of immunoglobulin fractions, and monoclonal antibodies, and as a potential for treatment of certain cancers and autoimmune diseases via extracorporeal plasma filtration.

Protein G is an immunoglobulin binding bacterial cell wall protein isolated from group G streptococci; it has recently been cloned and expressed in bacterial cell systems and hence is available in sufficient quantity to fully investigate its properties. Protein G is analogous to Protein A in that it too binds to the Fc region of immunoglobulins but is reported to be a more general IgG binding reagent since it will bind to certain animal IgGs and human IgG subclasses that interact poorly or not at all with Protein A. The Type III receptors (Protein G) are thought to have broader applications in the immunological arena than the Type I Fc Receptor (Protein A).

#### 1. Materials and Methods

Recombinant Protein A was purchased from Repligen Corporation (Cambridge, MA). Protein G (Type GXII$^{TM}$) was supplied by Genex Corporation (Gaithersburg, MD). Both proteins were immobilized to the new matrix as described in Tables X and XI. The affinity support used was activated to contain aldehyde groups at the end of an 18-atom spacer arm. The extended arm support was selected to prevent steric problems that sometimes occur when macromolecules are immobilized and

used to purify equally large macromolecules. All standard chemicals used in the immobilization or evaluation steps were reagent grade and purchased from typical laboratory suppliers. Purified bovine and human IgG fractions (Fraction II, III) were purchased from Sigma Chemical Co. (St. Louis, MO). Human serum was obtained from the American Red Cross (Farmington, CT) and was partially purified by ion exchange. Polyclonal rabbit antisera to human albumin and goat antisera to human IgG were purchased from Cone BioProducts (Seguin, TX) and were also partially purified by ion exchange before use. In one experiment, an affinity purified fraction of goat anti-human IgG was used. Laboratory peristaltic pumps (Rainin) were used during the coupling and testing of the affinity supports in order to achieve the flow rates required. On-line UV monitors (Gilson) were used to monitor the dynamic events. Protein measurements were routinely made by O.D. 280 and verified by Lowry.

Results and Discussion

Since little has been reported about the optimum coupling conditions for the ligands under study, two sets of experiments were performed. In the first set, as reported in Table X, both ligands were coupled via glutaraldehyde to an amino affinity support; in this case the ligand was stabilized by using sodium borohydride as the reducing agent. Comparative testing with various polyclonal IgGs consistently showed higher elution capacity from the Protein G column.

The results shown in Table XI establish a few additional points: (1) trimethylamineborane is also an acceptable reducing agent for Protein A and Protein G, (2) the new matrix is readily scaleable--a 2.5 X increase in column size and in amount of ligand coupled yield close to perfect scale-up in the amount of IgG that is eluted from both columns, and (3) the relatively weaker interaction of bovine species with Protein A is in agreement with published literature.

Although in general the data agrees with published literature, especially the weak interaction observed between goat and bovine IgGs

with Protein A, it should be kept in mind that both immobilized ligands are recombinant in nature, and therefore results may vary depending on the source and consistency of the cloned product. Total protein recoveries from all experiments ranged from 91-100%, suggesting that non-recoverable protein loss to the matrix is not a factor.

Table X

COMPARISON OF PROTEIN A AND PROTEIN G COLUMNS: INTERACTION WITH VARIOUS POLYCLONAL IgGs WITH SODIUM BOROHYDRIDE AS REDUCING AGENT

| | Protein A | | Protein G | |
|---|---|---|---|---|
| Species | Capacity (mg) | Ratio | Capacity (mg) | Ratio |
| Human IgG (pure) | 17.52 | 3.5 | 19.81 | 4.52 |
| Human IgG (serum) | 16.85 | 3.37 | 16.85 | 3.76 |
| Rabbit AHSA (antiserum) | 10.16 | 2.03 | 16.54 | 3.78 |
| Goat AHIgG (affinity purified) | 6.88 | 1.38 | 19.44 | 4.44 |

Coupling and Test Conditions: Laboratory-size columns containing 1 gram of Type A matrix (1,6-diaminohexane coupled matrix) were used. Columns were activated with 0.25% glutaraldehyde in 0.1M borate buffer, pH 8.2. After washing the columns free of excess glutaraldehyde, ligand solution (5 mg/ml) in the same buffer was recirculated for 4 hours. Columns were reduced with sodium borohydride ($NaBH_4$) and blocked with 1% glycine ethyl ester hydrochloride, pH 8.2. The blocking and reduction step was allowed to proceed overnight for convenience. After several washes alternating with buffer (0.05M sodium phosphate, pH 7.6, with 0.25M NaCl) and 0.2M glycine-HCl (pH 2.3), the columns were washed with 0.15M NaCl acidified to pH 2.0, then equilibrated with 0.05M sodium phosphate buffer with 0.25M NaCl, pH 7.6, prior to applying the IgG fractions. Flow rate throughout was 2 ml/min. To provide maximum contact time, all IgG solutions were recirculated for 1 hour. Elution of the IgG column was accomplished with glycine-HCl, pH 2.3. As determined by Lowry, the Protein A column coupled at 100% efficiency and contained 5 mg of immobilized ligand, whereas the Protein G column coupled at 88% efficiency and contained 4.4 mg of immobilized ligand. To provide consistent testing, the test solutions were adjusted (by dilution) so as to offer 50 mg (+/- 5 mg) total IgG to each column. Protein A and Protein G columns were tested simultaneously with the same starting pool of test sample to avoid the possibility of variation in the starting sample. The ratio provided above is defined as the interaction ratio between

total eluted IgG and immobilized ligand (i.e., eluted IgG/bound ligand).

Table XI

COMPARISON OF PROTEIN A AND PROTEIN G COLUMNS: INTERACTION WITH VARIOUS POLYCLONAL IgGs WITH TRIMETHYLAMINEBORANE AS REDUCING AGENT

| | Protein A | | Protein G | |
|---|---|---|---|---|
| Species | Capacity (mg) | Ratio | Capacity (mg) | Ratio |
| Human IgG (pure) | 43.99 | 3.55 | 46.66 | 4.42 |
| Human IgG (serum) | 39.03 | 3.15 | 40.05 | 3.79 |
| Bovine IgG (pure) | 25.19 | 2.03 | 44.44 | 4.20 |

Coupling and Test Conditions: Slightly larger columns containing 2.5 g of Amino type matrix of Table X were used to test the scale-up efficiency of the new matrix. Columns were activated and ligands coupled as described in Table X. In this study trimethylamineborane was used as the reducing agent. This reagent was first dissolved in 1 M acetic acid and then combined with glycine-ethyl ester hydrochloride (final concentration 1.5%, pH 6.0) to block unbound sites. Blocking and reduction were continued overnight. The columns were washed, equilibrated, and tested as described above. As determined by Lowry, the Protein A column coupled at 99% efficiency and in this case contained 12.4 mg of immobilized ligand, whereas the Protein G column coupled at 97% efficiency and contained 10.6 mg of immobilized ligand. To provide consistent testing, the test solutions were adjusted (by dilution) so as to offer 125 mg (+/- 5 mg) total IgG to each column. Protein A and Protein G columns were tested simultaneously with the same starting pool of test sample to avoid the possibility of variation in the starting sample. As in Table X, the Ratio reported above = Eluted IgG/bound ligand.

The matrix composition and coupling methods are compatible with both ligands. It is proposed, based on the results shown, that the bacterial Fc receptors are coupled to the solid support through non-critical primary amino sites, leaving the Fc receptor sites accessible for interaction with the Fc region of immunoglobulins. The arithmetic scale-up observed when columns containing 2.5 X more matrix and immobilized ligand were tested with human IgG demonstrates that larger devices with flow rates in the 100-1,000 ml/min range can be similarly fabricated.

Further support for the scale-up capabilities of the matrix was obtained when human IgG was coupled to a newer form of the matrix, this support differing in that it is pre-activated to the aldehyde form. (Preactivation was accomplished by reacting the glycidyl group of the cellulose bound GMA with a weak acid such as a dilute perchloric acid to produce the dihydroxy form, i.e., PHMA. The PHMA may then be reacted with periodate to give the aldehyde form.) Laboratory results yielded ligand immobilization capacities of 10 mg, 80 mg, and 3,000 mg for devices capable of flowing at 2 ml/min, 5 ml/min, and 125 ml/min. The largest affinity filter was capable of isolating more than 3 grams of IgG from goat anti-human anti-serum.

## Example 16

## Preparation of a Tangential Flow Cartridge with Immobilized Protein A

Protein A was immobilized to hexamethylenediamine derivatized matrix configured in a 250 mL (housing volume) tangential flow cartridge containing 13.8 gm of matrix. The cartridge was first equilibrated with 0.1 M borate buffer, pH 8.2 (about 2000 mL) then treated with 7 mL of a 10 mg/ml solution of protein A, pH 8.2, was recirculated through the cartridge overnight (21 hrs). The flow rate was about 100 mL/min for the first 2.5 hrs, 50 mL/min for the next 1.5 hrs and then 25 mL/min for the remaining time.

The cartridge was then stabilized by adding 5 mL of a 26% solution of glycine ethyl ester, pH=8.2, to the existing solution so the final concentration equals approximately 1% (dead volume is approximately 130 mL). Two portions of sodium borohydride (100 mg) were added 20 min. apart from one another and the solution was recirculated for an additional four hours. The cartridge was then washed with buffer, pH=8.2, and the eluant reserved as a control for protein determination.

The column was deactivated by recirculating 500 mL of a 1% glycine ethyl ester solution, pH=8.2, through the cartridge. Sodium borohydride (4 x 250 mg each) was then added at 30 minute intervals. After about 1-1/2 hours, the solution was allowed to recirculated at 4°C overnight. Then, four portions of sodium borohydride (400 mg) were added (at 45 min. intervals) and the solution allowed to recirculate for about four hours at room temperature.

The column was then washed with the following solutions:

- 680 ml 0.1M borate, pH=8.2
- 1140 ml 0.2M glycine-HCl, pH=2.3
- 450 ml 0.1M borate, pH=8.2
- 1140 ml 0.2M glycine-HCl, pH=2.3
- 450 ml pH 6.5 buffer
- 570 ml 0.2M glycine-HCl
- 570 ml pH 6.5 buffer

70 mg of protein A was coupled to the matrix based on the Lowry color test, as described above. A graph of the pressure differential ($\Delta p$) versus flow rate (ml/min) appears in Figure 26.

The tangential flow cartridge was then tested for HγG purification. Three experiments were conducted at various flow rates. HγG ws dissolved in 0.05 M phosphate buffer (5 mg/ml), pH 7.6 containing 0.25 M NaCl and recirculated at flow rates of 25, 100, and 140 mL/min for 60 minutes. As shown in Table XII, the capacity of the column was only slightly higher at 100 mL/min flow (208 mg) than at 50 mL/min (194 mg) and at 25 ml/min (195 mg).

TABLE XII

| Flow Rate (mls/min) | H&G Offered mg | Washing Vol ml | Elution Cap mg | Elution Vol | Inter. Ratio | Recovery % |
|---|---|---|---|---|---|---|
| 50 | 747 (5 mg/ml) | 1920 | 194 | 1500 | 2.78 | 93 |
| 100 | 683 (5 mg/ml) | 2000 | 208 | 1700 | 2.97 | 95 |
| 25 | 683 (5 mg/ml) | 2160 | 195 | 1700 | 2.78 | 95 |

In another experiment, undiluted plasma (70 mL), pH=7.8, was recirculated for 60 min at 75 ml/min. As shown in Table XIII, 98% recovery of IgG was observed.

| Total $O.D._{280}$ offered | Washing vol ml | Elution Cap (mg) (1) $O.D._{280}$ (2) Lowry ($O.D._{650}$) | Elution Vol (ml) | Inter. Ratio | Recovery % |
|---|---|---|---|---|---|
| 4061.4 | 4000 | (1) 298<br>(2) 146 | 2000 | (1) 4.25<br>(2) 2.09 | 98 |

In a third experiment, IgG within hemolysed blood (2 month old blood kept at 4°C) was purified. The tangential flow cartridge was first equilibrated with 0.3 NaCl, pH 7.8, then the cartridge is emptied and refilled with hemolysed blood (50 ml), pH=6.76 (unadjusted). The blood was then recirculated for 60 min at 75 ml/min and the IgG eluted. As shown in Table XIV, the amount of IgG eluted was 486.4 mg based on $O.D._{280}$ and 203 mg based on the Lowry method, $O.D._{650}$. As shown in Figure 27, the purity of the IgG was >95%.

A summary of the testing results appears in Table XIV.

Table XIV

SUMMARY OF TESTING RESULTS

| Protein A | Testing Conditions | Testing Substance | Elution Capacity | Inter. Ratio |
|---|---|---|---|---|
| (1) offered<br>(2) coupled | (1) Flow Rate<br>(2) pH and buffer<br>(3) contact time | | (1) $O.D._{280}$ based<br>(2) $O.D._{650}$ based | (1) $O.D._{280}$ based<br>(2) $O.D._{650}$ based |
| (1) 70 mg<br>(2) 70 mg (100%) | (1) 50 mls/min<br>(2) pH=7.6 with 0.25M NaCl, NaP<br>(3) 60 min | H G 747 mg (5 mg/ml) | (1) 194 mg<br>(2) -- | (1) 2.78 |
| (1) 70 mg<br>(2) 70 mg (100%) | (1) 100 mls/min<br>(2) pH=7.6 with 0.25M NaCl, NaP<br>(3) 60 min | H G 683 mg (5 mg/ml) | (1) 208 mg<br>(2) -- | (1) 2.97 |
| (1) 70 mg<br>(2) 70 mg (100%) | (1) 25 mls/min<br>(2) pH=7.6 with 0.25M NaCl, NaP<br>(3) 60 min | H G 682 mg (5 mg/ml) | (1) 194 mg<br>(2) -- | (1) 2.78 |

| Protein A | Testing Conditions | Testing Substance | Elution Capacity | Inter. Ratio |
|---|---|---|---|---|
| (1) 70 mg<br>(2) 70 mg<br>(100%) | (1) 75 mls/min<br>(2) pH=7.6 with<br>0.25M NaCl, NaP<br>(3) 60 min | 70 ml undiluted H. plasma, pH adjusted to 7.8 | (1) 298 mg<br>(2) 146 mg | (1) 4.25<br>(2) 2.086 |
| (1) 70 mg<br>(2) 70 mg<br>(100%) | (1) 75 mls/min<br>(2) 0.3 M NaCl<br>pH adjusted 7.8<br>(3) 60 min | 50 ml undiluted H. blood, pH 6.76 | (1) 486 mg<br>(2) 203 mg | (1) 6.94<br>(2) 2.9 |

## Example 17

### Comparison of Different Ligands on Adsorption of Heparin from Blood in Static Tests

Experimental media was placed in a test tube. Buffered saline, human plasma, and whole blood were made to contain 10 or 20 units/ml heparin (Sigma Heparin from Porcine Intestinal micosa H-7005; activity 150-180 USPK1 U (units)/mg). Test fluid was left in contact with media for 30 min, then samples were centrifuged for approximately 5 min at low speed, just until media was pelleted.

A colorimetric assay for heparin in plasma published by Michael D. Klein in Analytical Biochemistry 124:59-64 (1982) was used (see Example 13).

Graphs depicting the relationship between surface area and % heparin removal for the three affinity ligands (0.1 g media/10 $cm^3$, contain time = 1 hr, blood volume = 10 mL, heparin concentration 20 U, citrate concentration = 2.9 mg/mL) are depicted in Figure 19.

As shown in Figure 19, the high molecular weight polymer of ligand C (polyethyleneimine ligand) is the strongest heparin adsorbent. 100% of heparin removal is achievable by contacting 10 ml of blood containing 20 U/ml with 250 $cm^2$ media for 1 hour. Approximately 20% of the 29 mg citrate existing in the blood was also found to be adsorbed by the same media.

Next, the effect of contact time was determined. The results appear in Figure 20 which depicts the % heparin removal for various times using ligand A (hexamethylenediamine) and protamine.

Experiments were conducted by laminating a piece of Zetaffinity paper of size 7 cm x 7.5 cm or 52.5 $cm^2$ in a 150 ml blood bag. 680 U of heparin (45% x 150 ml x 10 U/ml) was found to be removed overnight with Ligand A, which corresponds to a media capacity of 13 U heparin removed per $cm^2$ paper.

The results depicted in Figure 20 show that approximately 1 hour

contact time is required to have 90% of the absorption sites utilized for binding heparin.

Next, the effect of protamine concentration on the amount of heparin removed from blood was determined. Various concentrations of ligand (0-200 mg protamine/g media) were contacted with 10 mL of blood having 20 U/mL heparin. The samples were allowed to react for 1 h then the amount of heparin bound determined as described above. A graph depicting the amount of heparin bound to the matrix for two sheets of matrix material having surface areas of 12 $cm^2$ and 6 $cm^2$ are shown in Figure 20.

## Example 17

## Preparation and Dynamic Testing of a Tangential Flow Cartridge for Heparin Removal

A tangential flow cartridge was constructed based on the "250 size" cartridge as shown in Figure 11. Webbing material as first wrapped around the core twice to prevent the media from blanking off the core. Then, the media and impermeable film are wrapped together with the webbing material until a diameter of 2.600 inches was obtained. The media and film were then cut and a 4-inch piece of webbing was inserted 2 inches between the media and the preceding wrap of webbing. Finally, the subassembly is held together with elastic bands and then capped with polypropylene end caps. The 250 mL size cartridge (housing volume) contains 14.4 grams of matrix with a total surface area of 180 $cm^2$.

The tangential flow cartridge was thoroughly flushed with saline solution at 50 ml/min. One unit of blood was doped with 10 U/ml heparin and pumped through the pre-flushed cartridge at a flow rate of 12 ml/min ($\Delta p$ = 1.5 psi). The filtered blood was fraction-collected at the last 10 ml per every 100 ml fraction. Heparin concentration in each fraction was analyzed by the colorimetric assay and plotted as shown in Figure 23. Figure 23 shows that approximately 70% of the

heparin was removed. In a separate experiment (data not shown), 80% of the heparin was removed by passing two units of blood through an 800 size cartridge.

The pressure differential across the tangential flow cartridge at flow rates between 0 and 160 ml/min for blood and saline was then determined. The results in Figure 22 show that for media having a length of 6'11", the pressure differential for blood increases more rapidly than for saline at increasing flow rates.

In Table XV, the analysis of blood components for various volumes of filtered blood for the 250 mL size tangential flow cartridge is depicted. Table XVI shows the analysis of blood components for various volumes of blood using the 800 mL size tangential flow cartridge.

Table XV

FILTRATION OF HEPARINIZED BLOOD BY TANGENTIAL FLOW CARTRIDGE SIZE 250

| Blood Component | Volume of Blood Filtered | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 100 | 200 | 300 | 400 | 500 |
| Hct ml/100 ml | 35.0 | 28.3 | 33.5 | 34.0 | 35.0 | 28.0 |
| Hb gm/d.1 | 10.9 | 9.5 | 10.8 | 11.0 | 10.6 | 8.9 |
| Hb in plasma | 15.15 | 4.34 | 9.61 | 11.16 | 9.3 | 7.13 |
| RBC x $10^6/\mu l$ | 3.59 | 3.04 | 3.49 | 3.59 | 3.43 | 2.56 |
| WBC x $10^3/\mu l$ | 5.177 | 3.446 | 4.231 | 4.475 | 4.525 | 3.970 |
| Plat count x $10^3/\mu l$ | 227 | 95.5 | 125.5 | 117.5 | 118 | 108.5 |
| Ext. Na/K | 170.8/4.35 | 167.3/2.67 | 163.8/3.96 | 167.0/4.25 | 169.7/4.35 | 166/3.1 |

Flow rate: 12 ml/min

p: 1.5 psi

Cartridge: Cross Flow, HDA, 250 size

Media wt = 14.4 g, L = 6'6"

Table XVI

FILTRATION OF HEPARINIZED BLOOD BY TANGENTIAL FLOW CARTRIDGE SIZE 800

| Blood Component | Volume of Filtered Blood (mL) 0 | 200 | 400 | 600 | 1000 |
|---|---|---|---|---|---|
| Hct ml/100 ml | 36.5 | 24.8 | 32.5 | 33.0 | 35.0 |
| Hb in blood | 12.6 | 8.5 | 11.5 | 12.0 | 12.4 |
| Hb in plasma | 37.9 | 25.5 | 37.9 | 35.6 | 37.2 |
| RBC x $10^6/\mu l$ | 3.78 | 2.64 | 3.49 | 3.58 | 3.59 |
| WBC x $10^3/\mu l$ | 3500 | 2145 | 2712 | 2846 | 2898 |
| Platelet count | 95 | 24 | 49 | 38 | 42 |
| Extra Na/K | 157.8/12.41 | 165.8/5.24 | 156.7/10.21 | 157.5/12.13 | 156.0/12.31 |

Cartridge: L = 26'

Flow Rate: 100 ml/min

The potential for affinity purification has been demonstrated on cartridge devices. Simplicity of use, and consistent scale-up capabilities, plus a matrix that is stable enough to be heat-sterilized before ligand attachment, provide possibilities for new approaches to the complex purification schemes presently used to isolate products from recombinant fluids. Availability of new ligands with improved specificity, such as shown with Protein A and Protein G, will further the advances being made in development of more process-oriented affinity supports.

Having now fully described this invention, it will be understood by those skilled in the art that the same can be performed within a wide and equivalent range of parameters, conditions, structures, and uses without effecting the spirit or scope of the invention or of any embodiment thereof.

WHAT IS CLAIMED AS NEW AND DESIRED TO BE SECURED BY LETTERS PATENT OF THE UNITED STATES IS:

1. A modified polysaccharide material which comprises:
   (1) a water-insoluble polysaccharide covalently bonded to a synthetic polymer;
   (2) said synthetic polymer made from
      (a) a polymerizable compound which has an epoxy group capable of direct covalent coupling to said polysaccharide; and
      (b) one or more polymerizable compounds containing
         (i) a chemical group capable of causing the covalent coupling of said synthetic polymer to an affinity ligand or a biologically active molecule, or
         (ii) a hydrophobic chemical group.

2. The material of claim 1 wherein said synthetic polymer is a homopolymer.

3. The material of claim 2 wherein said synthetic polymer is a homopolymer of glycidyl acrylate or glycidyl methacrylate.

4. The material of claim 1 wherein said synthetic polymer is a copolymer.

5. The material of claim 1 wherein said polysaccharide is cellulose.

6. The material of claim 1 wherein said chemical group capable of causing said covalent coupling has been reacted with an affinity ligand.

7. The material of claim 1 wherein said chemical group capable of causing the covalent coupling of said synthetic polymer to said affinity ligand is selected from the group consisting of a primary amine and an aldehyde.

8. The material of claim 6 wherein said affinity ligand is an enzyme, a nucleic acid, an antigen, an antibody, a saccharide, a lectin, an enzyme cofactor, an enzyme inhibitor or a binding protein.

9. The material of claim 8 wherein said ligand is selected from the group consisting of benzamidine, Protein A, Protein G, polymyxin-B, protamine and heparin.

10. A self-supporting cellulosic fibrous matrix which comprises the material of claim 6.

11. In a method of affinity chromatography, the improvement comprising utilizing as the insoluble ligand support the material of claim 1.

12. In a method of carrying out chemical reactions using an insolubilized biologically active molecule, the improvement wherein the insoluble support for said molecule is the material of claim 1.

13. The methods of any one of claims 11 or 12 wherein said material is in the form of a self-supporting cellulosic fibrous matrix.

14. A process for preparing the modified polysaccharide material of claim 1 which comprises:

(1) polymerizing said compound (a) which has an epoxy group capable of reacting with the hydroxy group of said polysaccharide, with said compound (b) in the presence

of said polysaccharide, under temperature conditions insufficient to cause the covalent binding of said compound (a) to said polysaccharide, to thereby form a synthetic polymer of (a) and (b);

(2) reacting said polysaccharide with said chemical group of compound (a) of said synthetic polymer under conditions sufficient to cause said covalent bonding.

15. A chromatography column for effecting chromatographic separation of at least two components of a sample flowing therethrough comprising:

a housing;

at least one solid stationary phase in said housing, comprising a matrix having chromatographic functionality and being effective for chromatographic means for distributing the sample through the stationary phase; and

means for collecting the sample after the sample has flowed through the stationary phase, wherein said matrix comprises the material of claim 1.

16. A chromatography column for effecting chromatographic separation of at least two components of a sample flowing therethrough comprising:

(1) a housing, said housing comprising:

(a) an inlet housing member, and

(b) an outlet housing member, said inlet housing member and said outlet housing member defining a radially, outwardly expanding stationary phase chamber; and

(2) a stationary phase within said radially outwardly expanding stationary phase chamber, said stationary phase chamber comprising a matrix having chromatographic functionality and being effective for chromatographic separation;

wherein said stationary phase and said radially outwardly expanding stationary phase chamber coact to provide substantially uniform radial distribution of sample across said stationary phase, wherein said matrix comprises the material of claim 1.

17. A chromatography column for effecting chromatographic separation of at least two components of a sample flowing therethrough comprising:

(1) a housing, said housing comprising:

(a) an inlet housing member, and

(b) an outlet housing member, said inlet housing member and said outlet housing member defining a stationary phase chamber; and

(2) a stationary phase within said stationary phase chamber, said stationary phase comprising a matrix having chromatographic functionality and being effective for chromatographic separation,

wherein said stationary phase chamber and said stationary phase coact to distribute sample across said stationary phase, said matrix comprising the material of claim 1.

18. A chromatography column for effecting chromatographic separation of at least two components of a sample flowing therethrough comprising:

(1) a housing, said housing comprising:

(a) an inlet housing member, and

(b) an outlet housing member, said inlet housing member and said outlet housing member defining a radially, outwardly expanding stationary phase chamber; and

(2) a stationary phase within said radially, outwardly expanding chamber, said stationary phase comprising:

(a) matrix having chromatographic functionality and being effective for chromatographic separation, wherein said station-

ary phase chamber and said stationary phase coact to distribute sample across said stationary phase, said matrix comprising the material of claim 1.

19. A process for effecting chromatographic separation of at least two components of a sample comprising contacting said sample with a self-supporting fibrous matrix comprising a modified polysaccharide material, said modified polysaccharide material comprising:

(1) a water insoluble polysaccharide covalently bonded to a synthetic polymer;

(2) said synthetic polymer comprising

(a) a polymerizable compound which has an epoxy group capable of direct covalent coupling to said polysaccharide; and

(b) one or more polymerizable compounds containing

(1) a chemical group capable of causing the covalent coupling of said synthetic polymer to an affinity ligand or a biologically active molecule, or

(ii) a hydrophobic chemical group.

20. The process of claim 19, wherein said contacting comprises radial flow of said sample through said matrix.

21. The process of claim 19, wherein said contacting comprises tangential flow of said sample across said matrix.

22. A chromatographic device for effecting chromatographic separation of at least two components of a sample, comprising:

(A) a modified polysaccharide material which comprises:

(1) a water-insoluble polysaccharide covalently bonded to a synthetic polymer;

(2) said synthetic polymer made from

(a) a polymerizable compound which has an epoxy group capable of direct covalent coupling to said polysaccharide; and

(b) one or more polymerizable compounds containing

(i) a chemical group capable of causing the covalent coupling of said synthetic polymer to an affinity ligand or a biologically active molecule, or

(ii) a hydrophobic chemical group; and

(B) means for effecting tangential flow of said sample across said modified polysaccharide material.

23. The device of claim 22, wherein said modified polysaccharide material is in the form of a sheet, a corrugated sheet, or tube.

24. A chromatography device for effecting chromatographic separation of at least two components of a sample, comprising:

(1) a cylindrical core,

(2) at least a first and second stationary phases wound around said cylindrical core, wherein

(a) a first phase comprises a modified polysaccharide material which comprises:

(i) a water-insoluble polysaccharide covalently bonded to a synthetic polymer;

(ii) said synthetic polymer made from

(1) a polymerizable compound which has an epoxy group capable of direct covalent coupling to said polysaccharide; and

(2) one or more polymerizable compounds containing

(a) a chemical group capable of causing the covalent coupling of said synthetic polymer to an affinity

ligand or a biologically active molecule, or

(b) a hydrophobic chemical group,

wherein said first phase is in the form of a sheet;

(b) a second phase comprising a means for supporting, separating, and providing channels tangential to said cylindrical core;

(3) means for distributing the sample to said channels;

(4) means for collecting the sample from said channels; and

(5) a cylindrical housing.

25. The device of claim 24, further comprising a nozzle disposed on the end of said cylindrical housing, wherein said means for collecting the sample from said channels comprises one or more grooves along the axial length of said cylindrical core which are in fluid communication with said channels and said nozzle.

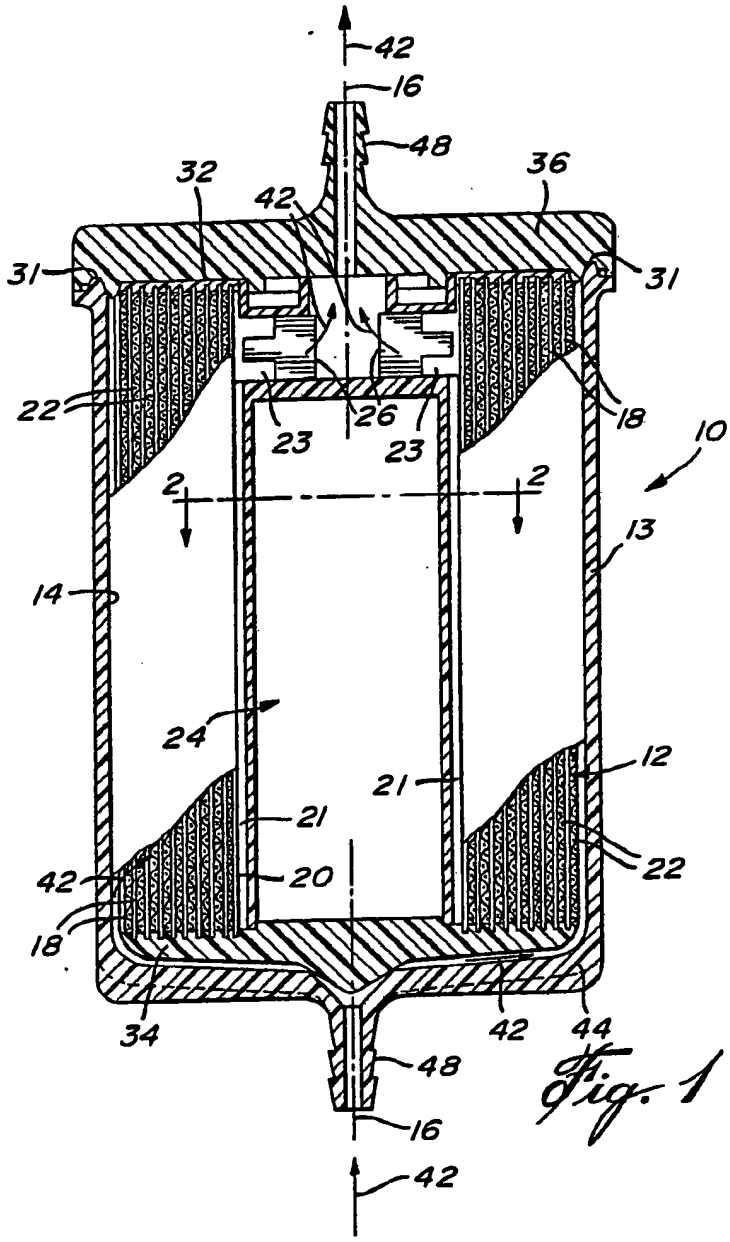
26. The device of claim 24, further comprising a nozzle disposed on the end of said cylindrical housing, wherein said means for distributing the sample to said channels comprises one or more grooves along the axial length of said cylindrical housing which are in fluid communication with said channels and said nozzle.

27. The device of claim 24, wherein said means for separating, spacing, and providing channels comprises two arrays of filaments, comprising

(1) a first array spaced parallel to one another and perpendicular to the axis of said cylindrical core, and

(2) a second array of filaments spaced parallel to one another and disposed at an angle to to said first array.

28. The device of claim 27, wherein said first array and said second array are affixed at their crossover points.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16
48
36
32
42
31
31
22
23
26
23
18
10
2
2
13
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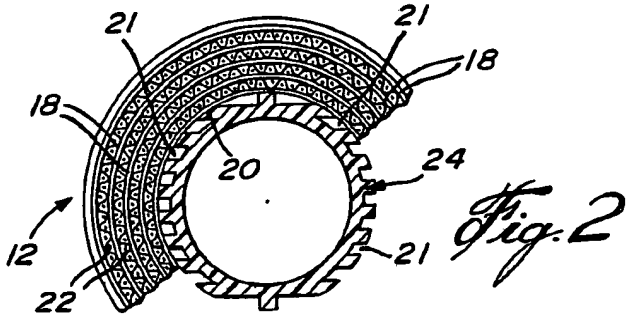
24
12
21
21
22
42
20
18
34
42
44
48
16
42

Fig. 1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21
18
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20
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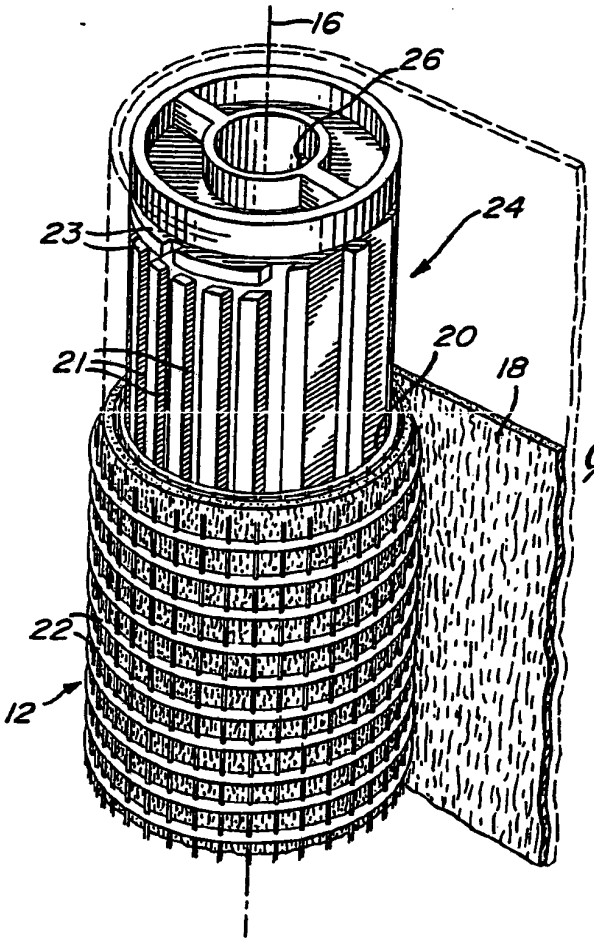
12
21
22

Fig. 2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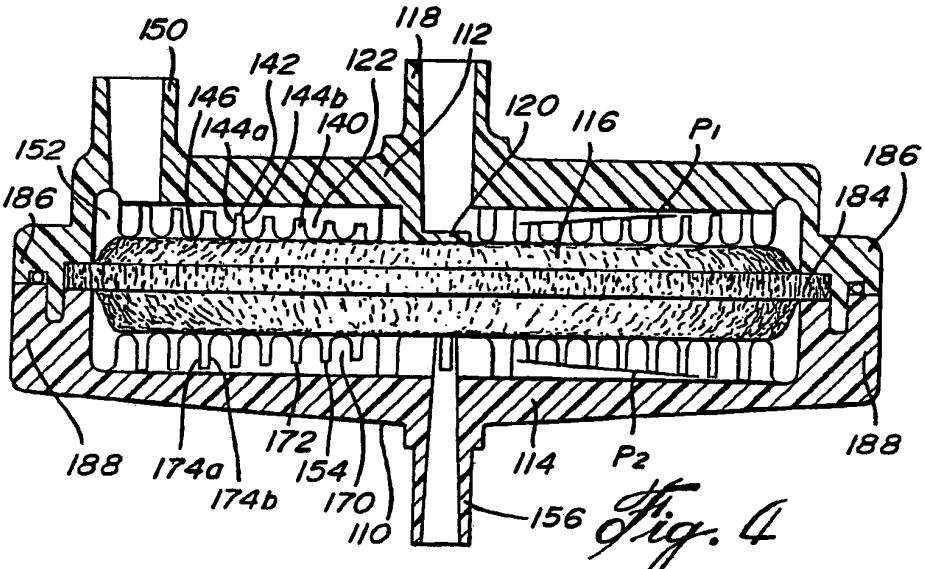
21
20
18
22
12

Fig. 3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118
112
142
122
146
144b
144a
140
120
116
P1
152
186
184
186
188
174a
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154
174b
170
110
114
P2
188
156

Fig. 4

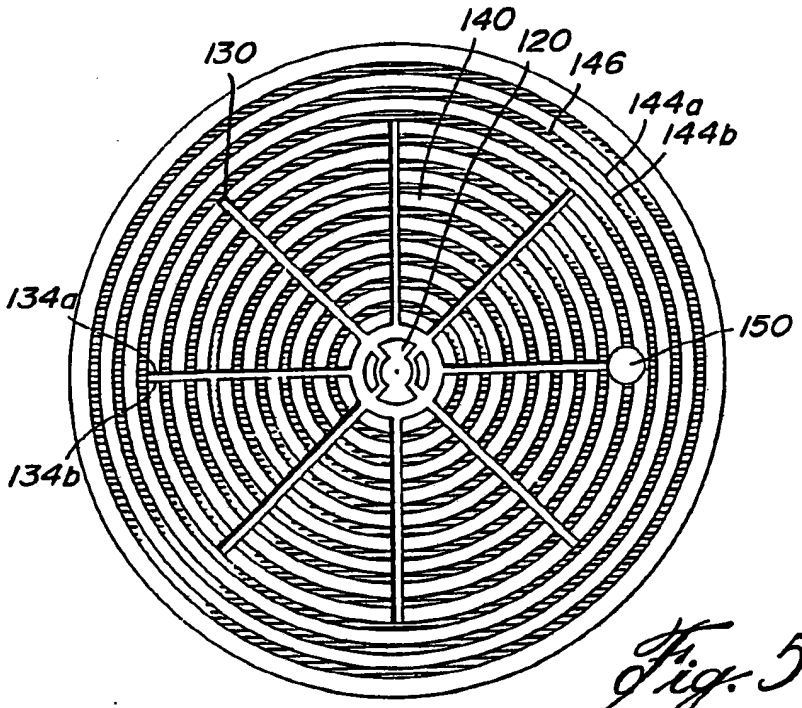



Fig. 5

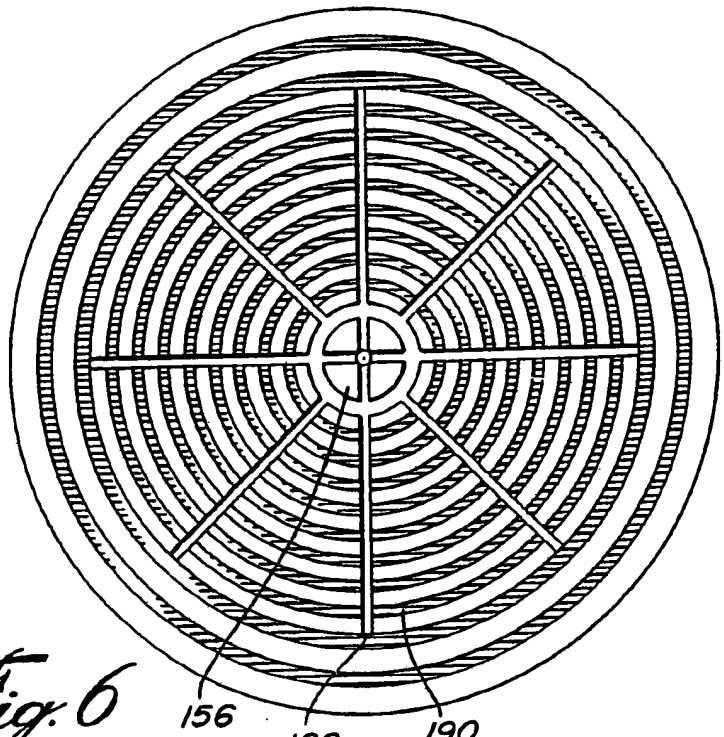



Fig. 6

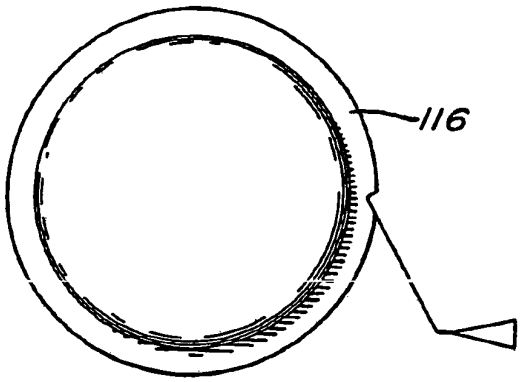
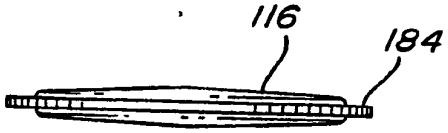



Fig. 7

Fig. 8



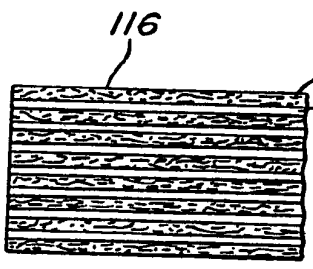




Fig. 9

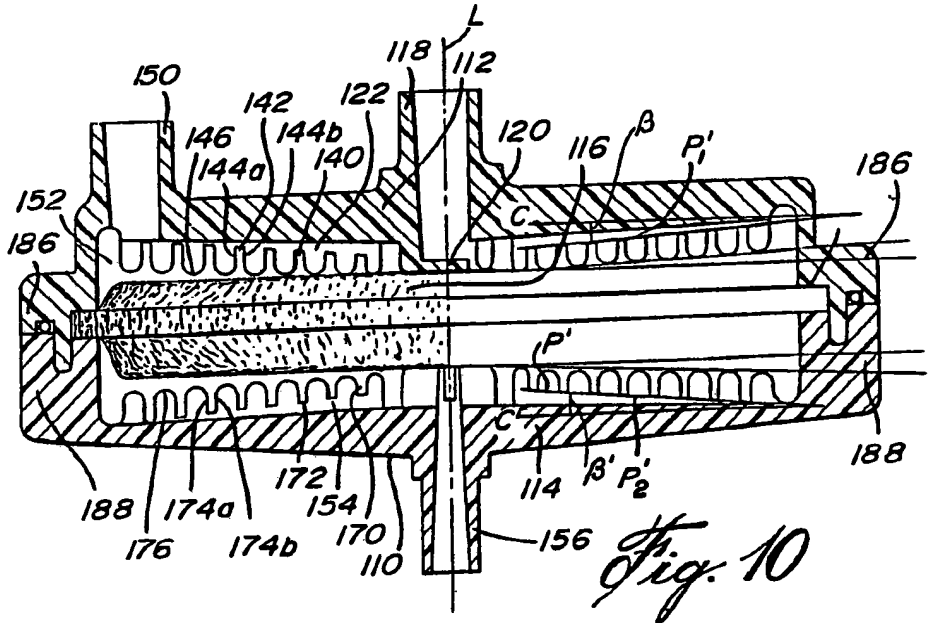

L
118
112
150
142
122
146
144b
120
116
β
P1'
186
144a
140
152
C
186
P'
C'
188
172
154
114
β'
P2'
188
174a
170
176
174b
110
156

Fig. 10

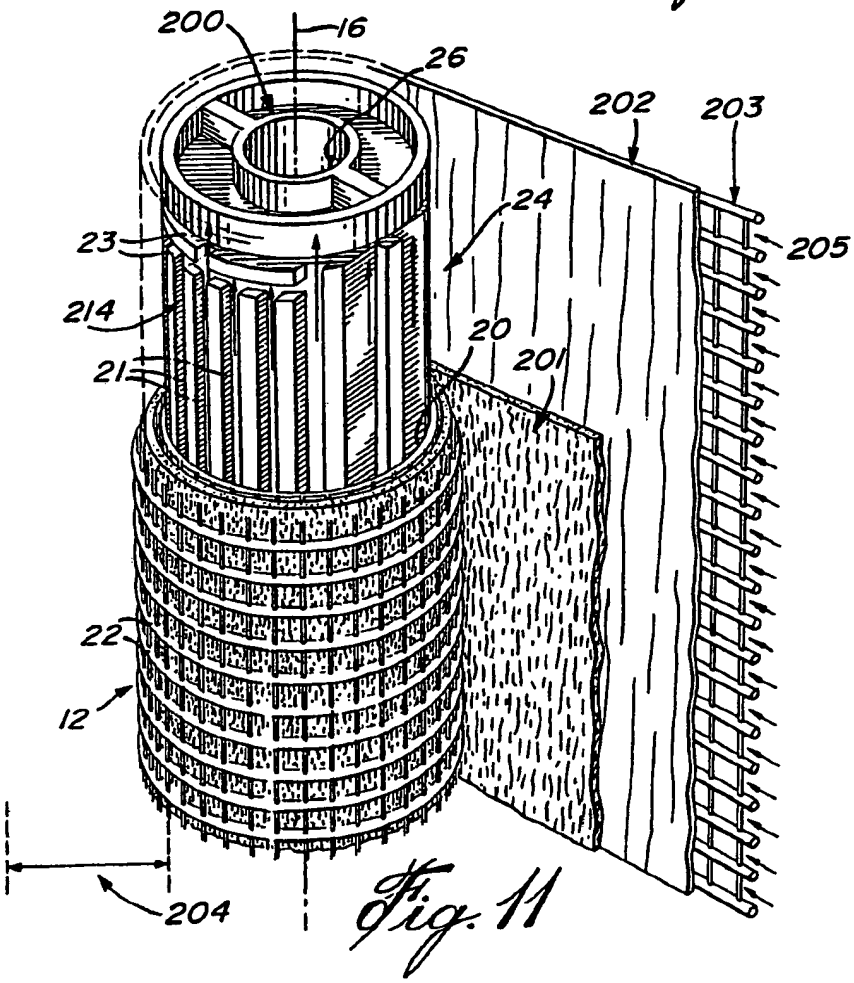

200
16
26
202
203
24
205
23
214
20
201
21
22
12
204

Fig. 11

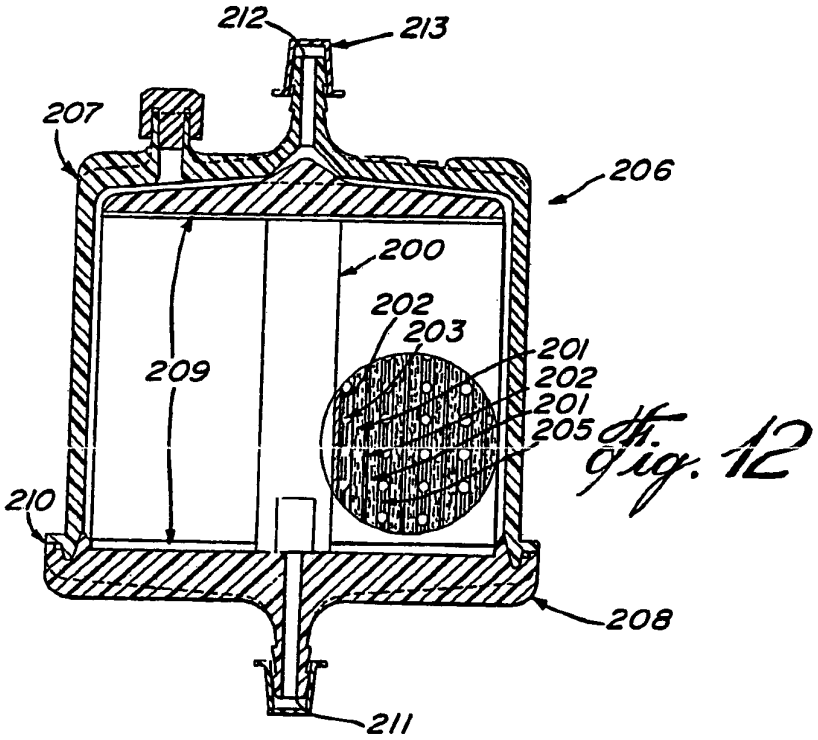



Fig. 12

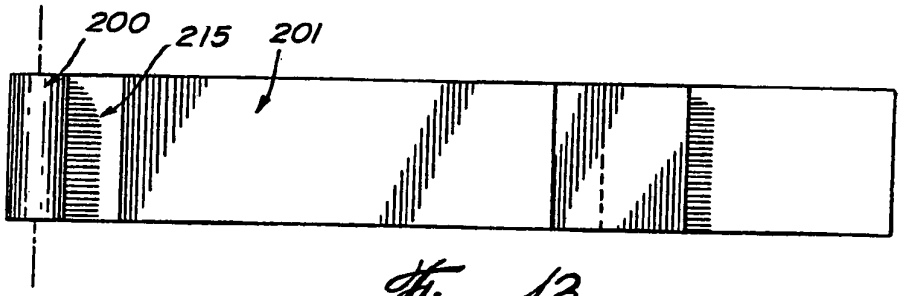



Fig. 13A

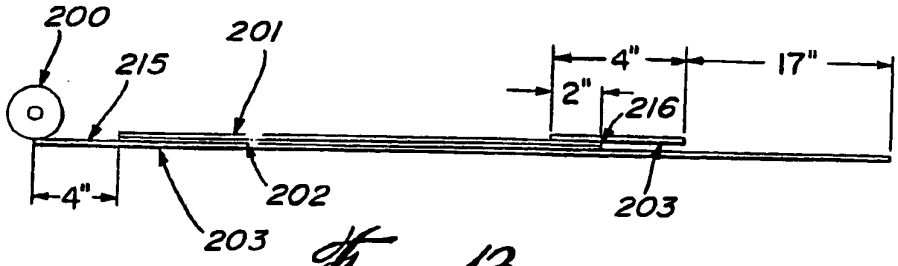



Fig. 13B

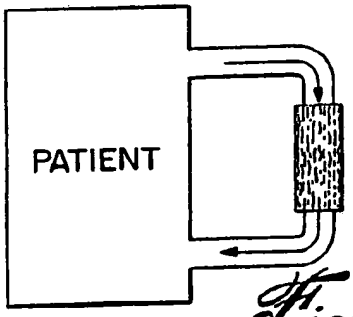



Fig. 14A

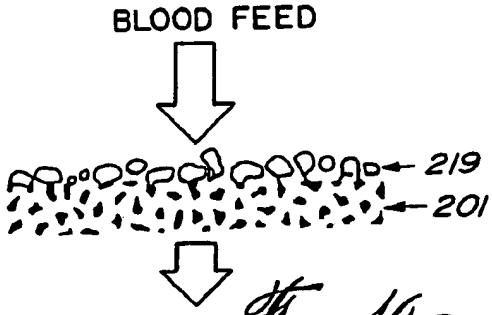



Fig. 14B

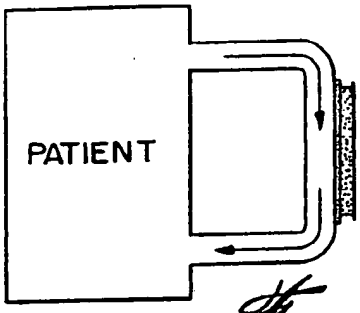



Fig. 14C

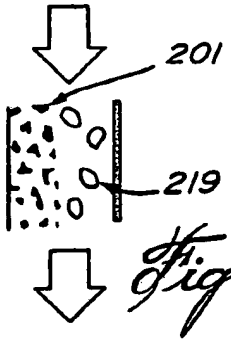



Fig. 14D

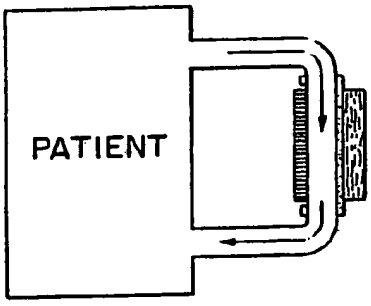



Fig. 15A

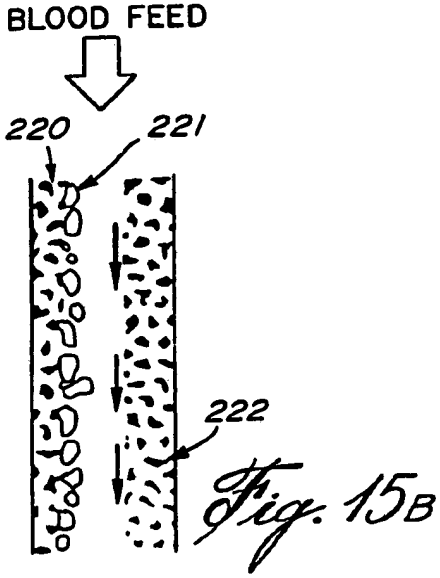
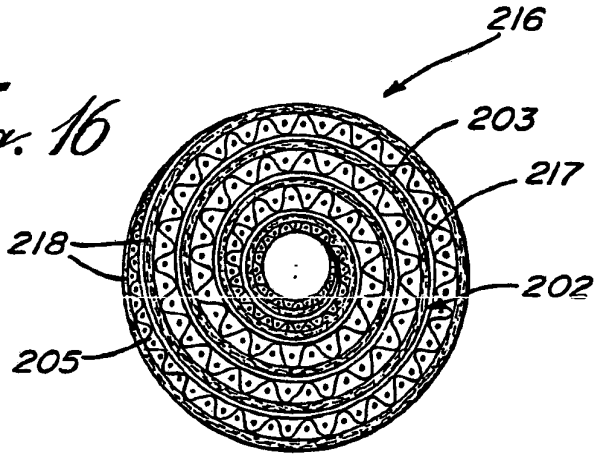



Fig. 15B

Fig. 16



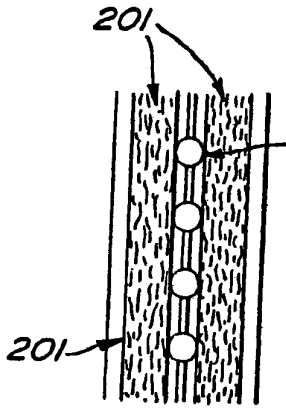
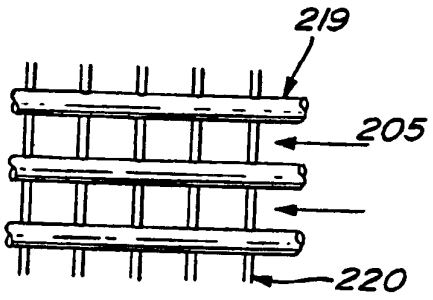




Fig. 17

Fig. 18



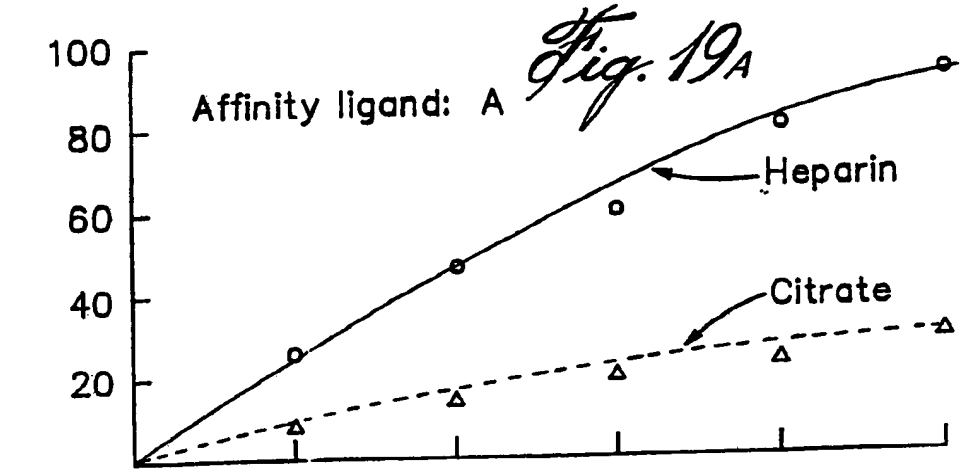



Fig. 19A

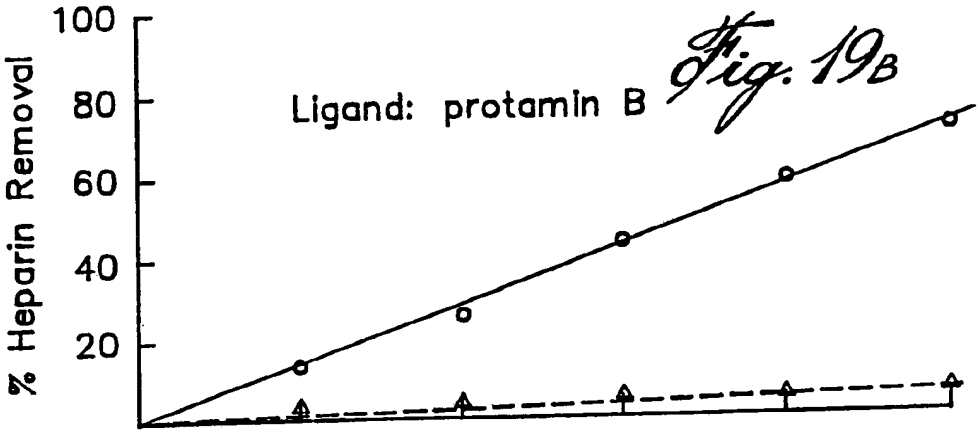



Fig. 19B

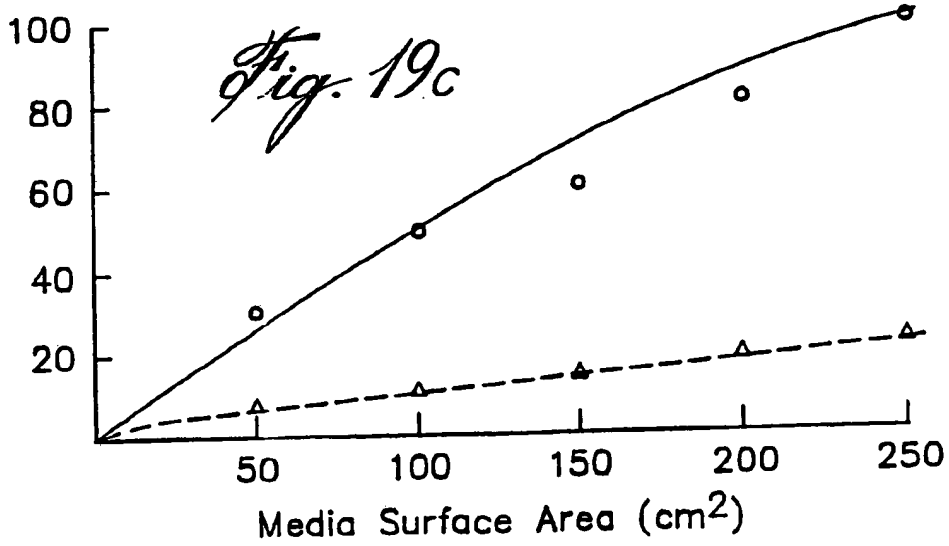



Fig. 19C

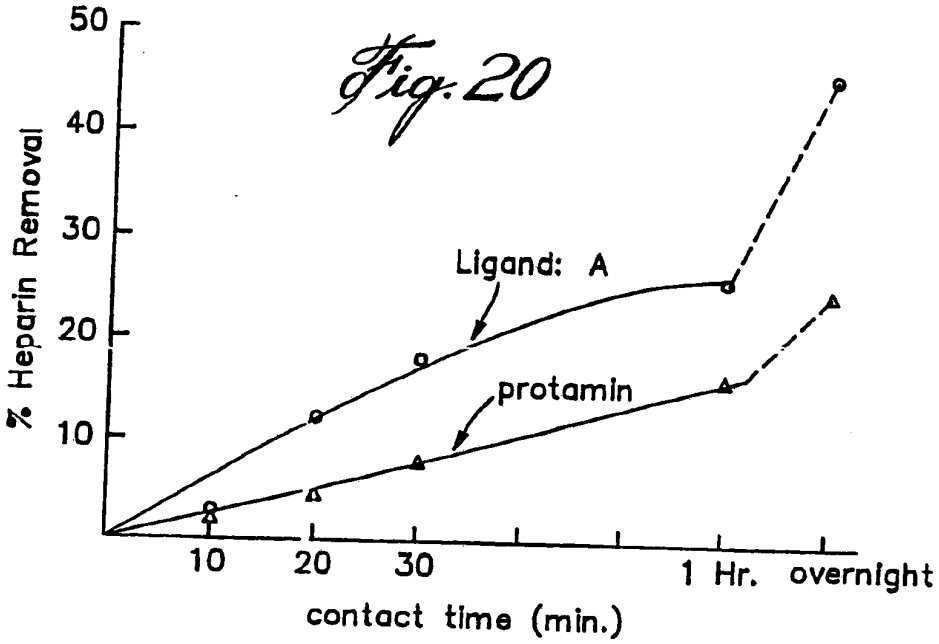

50
40
30
20
10
% Heparin Removal
Ligand: A
protamin
10
20
30
1 Hr.
overnight
contact time (min.)

Fig. 20

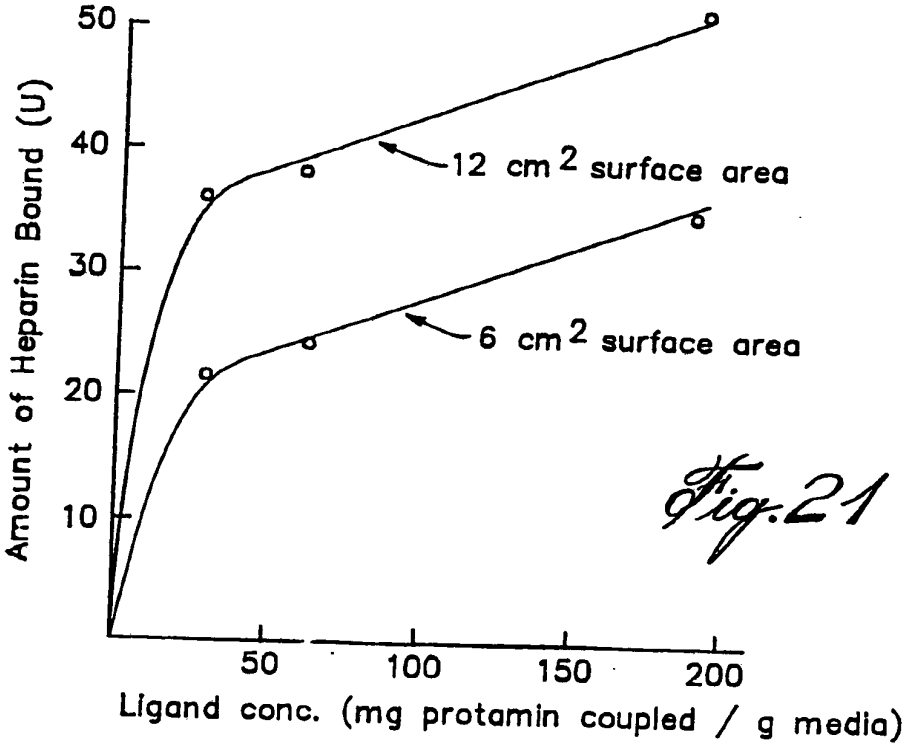

50
40
30
20
10
Amount of Heparin Bound (U)
12 cm 2 surface area
6 cm 2 surface area
50
100
150
200
Ligand conc. (mg protamin coupled / g media)

Fig. 21

Fig. 22

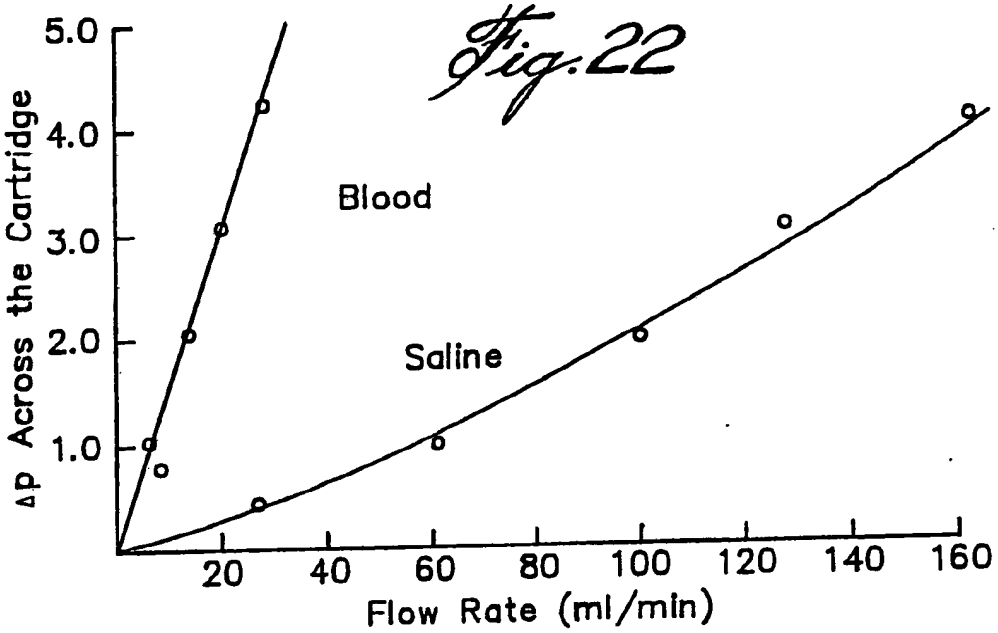
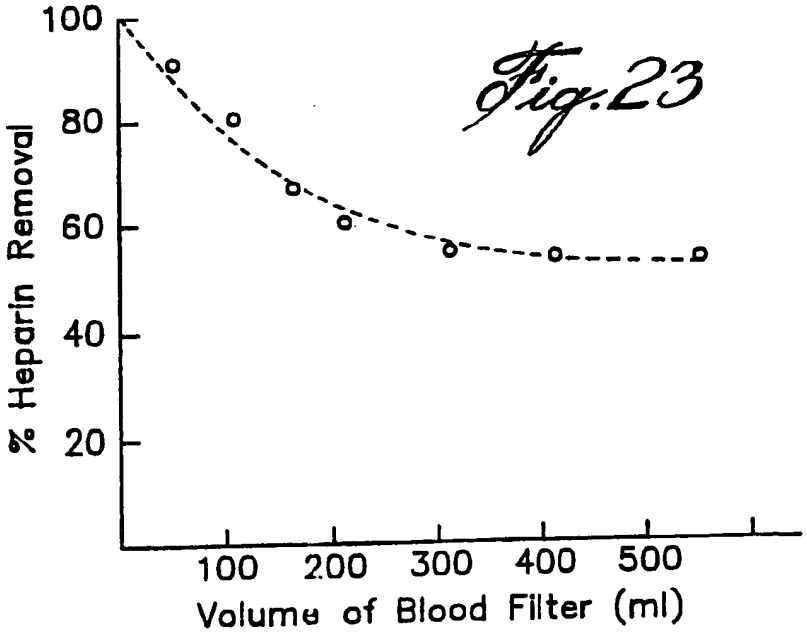

5.0
4.0
3.0
2.0
1.0
Δp Across the Cartridge
Blood
Saline
20
40
60
80
100
120
140
160
Flow Rate (ml/min)

Fig. 23


100
80
60
40
20
% Heparin Removal
100
200
300
400
500
Volume of Blood Filter (ml)

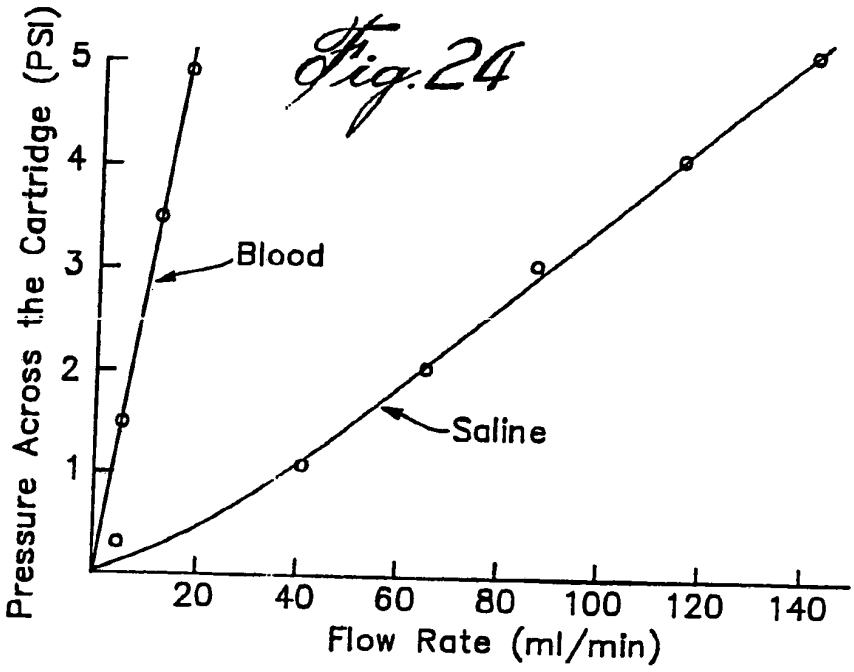

Pressure Across the Cartridge (PSI)
5
4
3
2
1
Blood
Saline
20
40
60
80
100
120
140
Flow Rate (ml/min)

Fig. 24

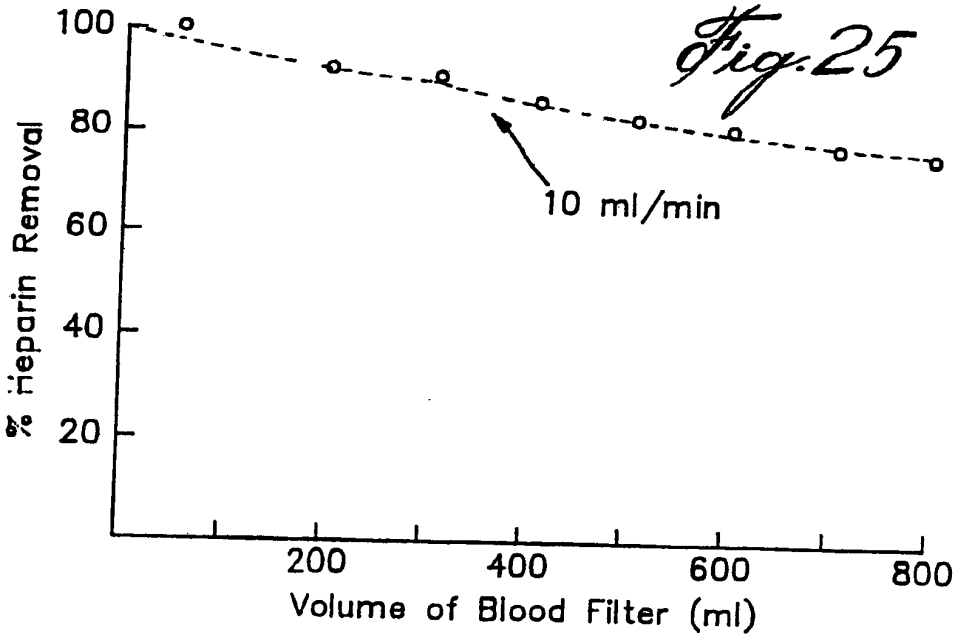

100
80
60
40
20
% Heparin Removal
10 ml/min
200
400
600
800
Volume of Blood Filter (ml)

Fig. 25

Fig. 26

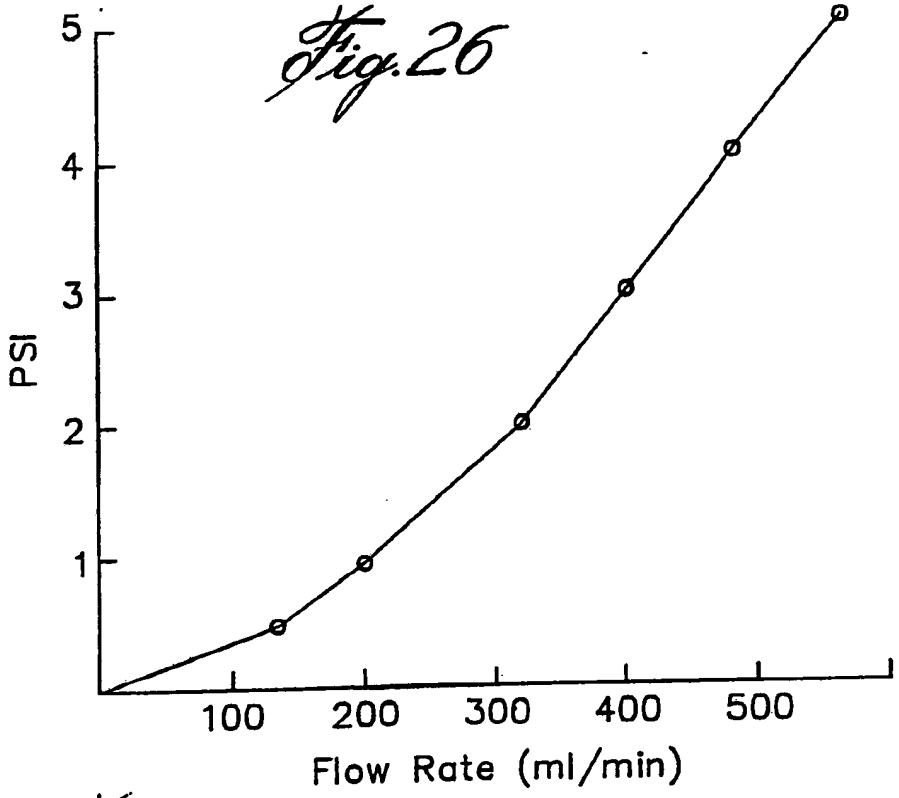
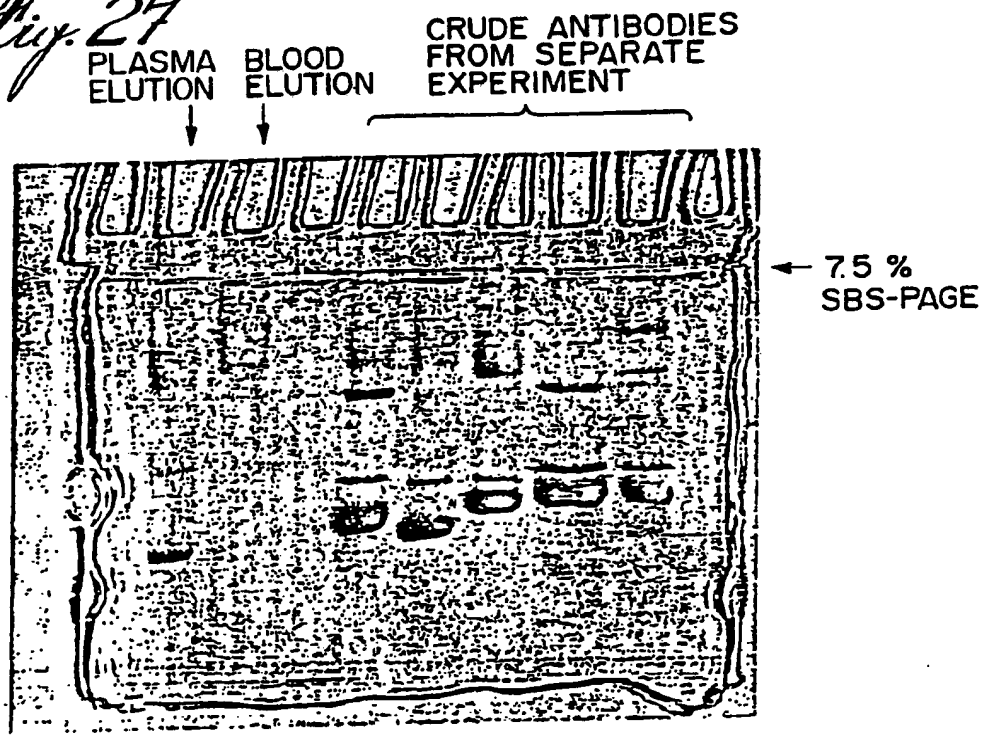



Fig. 27



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/00372

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl. 4th C08F 16/06;B01D 15/08;B01D 39/02;B01D 15/08; C12N 11/08; See Attachment

## II. FIELDS SEARCHED

| Minimum Documentation Searched 7 | |
|---|---|
| Classification System | Classification Symbols |
| U.S. | 525/54.2, 210/198.3; 210/502; 210/635; 210/656; 435/180 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched 8

Online research conducted on chemical abstracts and biosis.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

| Category * | Citation of Document, 11 with indication, where appropriate, of the relevant passages 12 | Relevant to Claim No. 13 |
|---|---|---|
| y | US, A, 4,224,439, Ayers et al, Published 23 September 1980 (Column 1-2) | 1-14 and 19-21 |
| y | US, A, 4,421,650, Nagasawa et al, published 20 December 1983 (See abstract) | 1-14 and 19-21 |
| y | US, A, 4,385,991, Rosevear et al, published 31 May 1983 (See abstract) | 1-14 and 19-21 |
| y | US, A, 4,352,884, Nakashima et al, published 05 October 1982 (See the entire document) | 1-14 and 19-21 |
| P,X | US, A, 4,743,373, Rai et al, published 10 May 1988 (see figures 1-10 and column 7, lines 14-46) | 15-18 and 22-28 |
| y | US, A, 4,384,957 Crowder, III et al, published 24 May 1983 (See the entire document) | 15-18 and 22-28 |

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 27 March 1989 | 05 JUL 1989 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| ISA/US | Abdel A. Mohamed<br>Adbel A. Mohamed |